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Pentraxin 3 promotes oxLDL uptake and inhibits cholesterol efflux from macrophage-derived foam cells ☆

Weishuo Liu [a,b], Jianwei Jiang [c], Dan Yan [a], Dujuan Li [a], Wei Li [a], Yungui Ma [a], Lili Yang [a], Zhiling Qu [a], Qiurong Ruan [a,*]

[a] Institute of Pathology of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[b] Department of Pathology of the First Affiliated Hospital of Soochow University, Suzhou, China
[c] Department of Hepatobiliary Surgery, Union Hospital, Tongji Medical College, Huazhong Umversiy of Science and Technology, Wuhan, China

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ABSTRACT

Background: The objective of this study was to determine the effects of pentraxin3 (PTX3) on human oxidized low density lipoprotein (oxLDL) uptake and cholesterol efflux from human macrophage foam cells, which may play a critical role in atherogenesis.
Methods: The effects of PTX3 on oxLDL uptake and cholesterol efflux were determined after transfection of human THP-1 macrophages with pSG5hPTX3 or PTX3siRNA plasmids. To evaluate the role of specific signaling pathways, human THP-1 cells were pre-treated with inhibitors of the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), phosphatidylinositide 3-kinases (PI3-K), and p38 mitogen-activated protein kinase (MAPK) pathways (PD98059, LY294002, and SB203580, respectively), and then exposed to oxLDL for the uptake assay or oxLDL and [^{3}H]-cholesterol and apolipoprotein A-I (apoA-I) for the cholesterol efflux assay.
Results: PTX3 overexpression not only promoted oxLDL uptake but also significantly reduced cholesterol efflux to apoA-I; it also significantly decreased the expression of peroxisome proliferator-activated receptor-γ (PPARγ), liver X receptor alpha (LXRα) and ATP-binding membrane cassette transporter A-1 (ABCA1), which was increased with PTX3 silencing. Furthermore, PTX3 significantly increased p-ERK1/2 levels in THP-1-derived foam cells, and inhibition of ERK1/2 by PD98059 significantly reduced the oxLDL uptake and promoted the cholesterol efflux induced by PTX3 overexpression.
Conclusion: Here, we demonstrate that PTX3 affects lipid accumulation in human macrophages, increasing oxLDL uptake and inhibiting cholesterol efflux. That is the underlying possible mechanisms of PTX3 contribution to the progression of atherosclerosis.

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Introduction

It is well documented that immunological and inflammatory processes play a fundamental role in atherogenesis (Klingenberg and Hansson, 2009; Libby et al., 2010; Zwaka et al., 2001). Pentraxins, a superfamily of acute-phase proteins highly conserved during evolution and characterized by a multimeric, usually pentameric structure, are key components of the humoral arm of the innate immune system (Bottazzi et al., 2010; Norata et al., 2009, 2010). The pentraxin family is composed of the short pentraxins, such as C-reactive protein (CRP) and serum amyloid P component (SAP), and long pentraxins, including pentraxin 3 (PTX3). PTX3 is structurally related but distinct from CRP and SAP in terms of gene organization and localization, ligand recognition, cellular source, and inducing signals. Unlike CRP, PTX3 is not synthesized in the liver (Lee et al., 1994), but is released by monocytes/macrophages, endothelial cells, smooth muscle cells, adipocytes, and neutrophils in response to inflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α, as well as acetylated, oxidized, and enzymatically modified LDL.

The presence of PTX3 protein was demonstrated in the advanced atherosclerotic plaques and myocardial tissues of patients with acute myocardial infarction (AMI) by immunohistochemistry (Nebuloni et al., 2011; Rolph et al., 2002; Savchenko et al., 2008). In addition, modified atherogenic lipoproteins induced expression of PTX3 by human vascular smooth muscle cells (Klouche et al., 2004). Moreover, elevated plasma PTX3 levels were detected in patients with unstable angina pectoris (Inoue et al., 2007), and in the coronary artery at sites distal from the plaque lesion, PTX3 levels were significantly elevated compared with proximal sites, suggesting that it originated from the atherosclerotic plaque itself and may reflect active atherosclerosis (Inoue et al., 2007). In addition, PTX3 may represent an early marker

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* Corresponding author at: Institute of Pathology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China. Fax: +86 27 83650729.
E-mail address: ruanqiurong@sina.com (Q. Ruan).

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of myocardial lesion; higher PTX3 levels (>10.73 ng/mL) were associated with increased 3-month mortality in patients with AMI (Latini et al., 2004; Peri et al., 2000). Taken together, these data suggest that PTX3 may be involved in the pathogenesis of atherosclerosis.

The differentiation of monocytes to macrophages that accumulate oxLDL to form foam cells in the vessel wall represents a major event in the progression of atherosclerosis (Li and Glass, 2002; Ohashi et al., 2005). The uptake of extracellular lipid depots into macrophages is mediated by scavenger receptors, including CD36. In addition to oxLDL uptake, cholesterol efflux may play a pivotal role in the removal of excess cholesterol from extra hepatic cells, including macrophages and smooth muscle cells (Wang et al., 2008). The efflux of internalized cholesterol to apoA-I containing particles is mediated by cholesterol exporters, including ATP-binding cassette transporter (ABCA1), and scavenger receptor BI (SR-BI). Thus, decreased cholesterol efflux from the arterial wall may potentially promote the progression of atherosclerosis.

The objective of this study was to examine the hypothesis that PTX3 could directly alter oxLDL uptake and cholesterol efflux from macrophage foam cells. PTX3 overexpression inhibited cholesterol efflux via activation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) but not phosphatidylinositide 3-kinases (PI3-K) or p38 mitogen-activated protein kinases (MAPKs) as well as down-regulated the expression of the key cholesterol transporter, ABCA1, and nuclear transcription factors, peroxisome proliferator-activated receptor-γ (PPARγ) and liver X receptor alpha (LXRα).

Methods

Chemicals and reagents

PD98059, Ly294002, and SB203580 were purchased from Calbiochem (San Diego, CA, USA). Phorbol 12-myristate 13-acetate (PMA) and apolipoprotein A-I (apoA-I) were obtained from Sigma (St Louis, MO, USA). [1α, 2α (*n*)-^{3}H]-Cholesterol was purchased from Perkin-Elmer Life Sciences (Piscataway, NJ, USA). Human oxLDL and 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindo-carbocyanine percholate-labeled oxLDL (Dil-oxLDL) were obtained from Yiyuan Biotechnologies (Guangzhou, China). Rabbit polyclonal anti-PTX3 (NBP1-55588) and anti-ABCA1 (NB400-105) antibodies were obtained from Novus Biologicals (Littleton, CO, USA). Anti-ERK1/2 (9102), anti-phospho ERK1/2 (9101S), anti-p38 (9212), and anti-phospho p38 (9211S) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA); anti-PPARγ (sc-7273) and anti-LXRα (sc-13068) antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). Anti-β-actin monoclonal antibody (A-5316), the internal standard control, was purchased from Sigma.

Cell culture

Human THP-1 cells were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium containing 10% FBS (Gibco) at 37 °C in a humidified 5% CO_2 atmosphere for 3–4 days before prior to use. The cells were washed twice with RPMI 1640, and 1×10^6 cells/well were plated into 6-well plates, and differentiated to macrophage-like cells by pre-incubation with 160 nM PMA for 24 h. Viability of the cells was >95% throughout the experimental period as determined by trypan blue exclusion.

Transient transfection

PMA-differentiated THP-1 cells were grown in 6-well plates until reaching approximately 80% confluence after which the medium was changed to OptiMEM (Invitrogen) for transfection with either pSG5hPTX3 plasmids or PTX3siRNA using Fugene 6 reagent (Promega, Madison, WI, USA) at the indicated concentration (1.5 μg plasmid in 5.25 μL reagent). After 24 h, THP-1 cells were then pretreated with or without inhibitors (20 μM PD98059, 10 μM Ly294002 or 10 μM SB203580) for 30 min prior to the addition of 50 μg/mL oxLDL for 24 h as described previously (Jing et al., 2000; Suzuki et al., 2013; Yamaguchi et al., 2006).

The full-length human PTX3 expression vector (PSG5hPTX3) was kindly provided by Dr. Barbara Bottazzi (Rozzano, Milan, Italy). The pSG5 empty vector was used as a control. A PTX3-specific siRNA was amplified using the following primers (Invitrogen): forward primer, 5′-CAAAGAGGAAUCCAUAUGA dTdT-3′ and reverse primer, 5′-UCAU AUGGAUUCCUCUUUG dTdT-3′. The PTX3 siRNA or the negative control RNA (pGCsi.U6/neo/GFP RNAi-NC vector) were transfected into THP-1 cells using Fugene HD 6 reagent (Roche, Mannheim, Germany) for 24 h. To estimate PTX3siRNA transfection efficiency, a construct expressing GFP was routinely used, and its expression was visualized using fluorescence microscopy. The transfection efficiency of THP-1 cells ranged from 30 to 50%.

Isolation of LDL and preparation of oxLDL

OxLDL was prepared using a kit and following the manufacturer's instructions (Cat No: YB-002; Yiyuan Biotechnologies, Guangzhou, China). Briefly, human LDL (Cat.No.YB-001; Yiyuan Biotechnologies) was purified to homogeneity via ultracentrifugation (1.019 to 1.063 g/cc) and oxidized using 5 μM $CuSO_4$ in PBS at 37 °C for 20 h. Oxidation was terminated by adding excess EDTA-Na2, which was filter sterilized using a 0.22-μm filter, and stored in a solution containing PBS (pH 7.4) and 5 μM EDTA-Na2 at 4 °C.

Protein content was determined using the Lowry method, and the purity and charge of both LDL and oxLDL were evaluated by examining their electrophoretic migration in agarose gels. The degree of oxidation was determined by measuring the amount of thiobarbituric acid reactive substances (TBAR). LDL had TBAR values of <1 nmol/mg; oxLDL had TBAR values of >10 and <30 nmol/mg. All lipoproteins were used for experiments within 4 weeks after preparation.

Fluorescence microscopy

THP-1 cells (1×10^6 cells/well) were plated onto 6-well tissue culture plates and differentiated to macrophage-like cells by pre-incubation with 160 nM PMA in RPMI 1640 medium containing 10% FBS for 24 h at 37 °C in 5% CO_2. Cells were then transfected with the control or pSG5hPTX3 expression vectors for 24 h; 10 μg/mL Dil-oxLDL was added for the last 6 h in accordance with manufacturer's instruction manual. Plates were washed twice in PBS, and the fluorescence intensity of the cells was analyzed under an inverted fluorescent microscope (ZEISS-SIP No. MIC01774) along with Image Pro Plus image analysis software. Data are shown as mean fluorescence of 500 cells per treatment as previously described (Choi et al., 2005), with three fields analyzed per experiment. Three independent experiments were performed.

RT-PCR and real-time RT-PCR analysis

Total RNA was isolated from cells using Trizol reagent (TaKaRa). *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PTX3, PPARγ, LXRα, ABCA1, SR-BI*, and *CD36* gene expression was analyzed using the primers shown in Table 1 (Invitrogen) and amplification with an Applied Biosystems Thermal Cycler (Carlsbad, CA, USA).

GAPDH, PTX3, PPARγ, LXRα, ABCA1, SR-BI, and CD36 mRNA levels were also analyzed by real-time PCR using the SYBR Green Master (Toyobo) with gene-specific primers using a Stratagene Mx3000P system (La Jolla, CA, USA) according to the manufacturer's guidelines. GAPDH or β-actin served as an internal standard. Relative gene expression was calculated using the ΔΔCt method using the following equation: $2^{-\Delta(\Delta Ct)}$. $\Delta C_t = C_{t(\text{specific transcript})} - C_{t(\text{housekeeping transcript})}$ and $\Delta(\Delta C_t) = \Delta C_{t(\text{treatment})} - \Delta C_{t(\text{control})}$ as previously described (Rubic and Lorenz, 2006).

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